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The synthesis of the ribosomal proteins of *Escherichia coli* during stationary growth phase

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The synthesis of the ribosomal proteins of *Escherichia coli* during stationary growth phase

Abstract:

The repression of cell growth as a response to starvation leads the bacterial culture to enter stationary growth phase. The cell needs to conserve the energy to survive the starvation. As a result, the metabolic processes of the cell slow down. The ribosome is one of the most energy-consuming organelles, as its main function is to carry out the protein synthesis. The ribosomal proteins have a profound effect on the correct functioning and assembly of the ribosome. To determine whether or not the ribosomal proteins of *Escherichia coli* are synthesised during stationary growth phase, the changes in the protein quantities over 14 days after the beginning of the growth were calculated. 24 out of 54 ribosomal proteins were found to be synthesised during stationary growth phase. Many of these proteins were found to have a positive effect on the cell during stationary growth phase, such as maintenance of the ribosome structural integrity, for instance, via protein exchange, and self-downregulation of the ribosomal protein synthesis.

Keywords: *Escherichia coli*, ribosome, ribosomal proteins, protein synthesis, stationary growth phase

CERCS: P320; Nucleic acids, protein synthesis

Escherichia coli Ribosoomivalkude süntees statsionaarses kasvufaasis

Lühikokkuvõte:

Rakkude kasvu aeglustumisel stressitingimustel siseneb rakukultuuri statsionaarsesse kasvufaasi. Selle tulemusena aeglustub rakkude metabolism ning väheneb nende energiakasutus. Ribosoomid on ühed suurimatest energiatarbijatest rakkudes, viies läbi valkude sünteesi ehk translatsiooni. Ribosoomivalgud osalevad ribosoomi tertsiaalstruktuuri korrektses moodustumises ja funktsionaalsuse tagamises. Selleks, et detekteerida *Escherichia coli* ribosoomivalkude sünteesi statsionaarse kasvufaasi käigus, kasvatati rakke 14 päeva ning mõõdeti valkude koguse muutust rakukultuuris. Tulemused näitasid, et 24 r-valku 54st sünteesiti rakkudes statsionaarse kasvufaasi käigus. Sünteesitud r-valgud võivad omada positiivset efekti rakkudele, alustades ribosoomivalkude sünteesi repressiooniga ning

lõpetades olemasolevate ribosoomide struktuuri terviklikkuse hoidmisega, läbi r-valkude vahetuse.

Võtmesõnad: *Escherichia coli*, ribosoom, ribosoomivalgud, valkude süntees, statsionaarne kasvufaas

CERCS: P320; Nukleiinhappesüntees, proteiinisüntees

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TERMS, ABBREVIATIONS AND NOTATIONS

HPF - the hibernation promoter factor

LB - Luria Bertani medium

MOPS - 3-(*N*-morpholino)propanesulfonic acid

mRNA - messenger RNA

ppGpp - guanosine 3'-diphosphate 5-' diphosphate

RaiA - ribosome-associated inhibitor A

RelA - GTP pyrophosphokinase

RMF - ribosome modulation factor

RNA - ribonucleic acid

r-protein - ribosomal protein

rRNA - ribosomal RNA

SILAC - Stable Isotope Labeling by/with Amino acids in Cell culture

tRNA - transfer RNA

INTRODUCTION

One of the most energy-consuming organelles of the cell is the ribosome, function of which is to carry out the protein synthesis, or translation. The ribosome of *Escherichia coli* is a complex that is composed of proteins and ribonucleic acid (RNA) molecules, assembled into two coherent ribosomal subunits. The interactions between the ribosomal proteins (r-proteins) and the ribosomal RNAs (rRNAs) are necessary for the assembly and the functioning of the ribosome. However, the individual functions of such interactions are still being explored.

The growth of bacteria might be inhibited in response to the exhaustion of the nutrients in the medium (Kolter *et al*., 1993). As a result of such repression of growth, bacterial culture enters stationary growth phase that is represented as a plateau in the growth curve. The cells need to save energy to survive the starvation. As a result, most of the metabolic processes of the cell become less active. The translation is one of the most energy and resource-consuming processes in the cell (Pletnev *et al*., 2015). Therefore, during stationary growth phase, a rapid decrease in the level of protein synthesis is necessary due to the deficiency of amino acids in the bacterial cell (Pletnev *et al*., 2015). The cell has to regulate the expression of the genes already at a transcription level, by suppression of the "unnecessary" genes, that would save up the energy for survival (Pletnev *et al*., 2015).

The assembly of the new ribosome is one of the main reasons why the r-proteins are synthesised. However, they also might have extra ribosomal functions. This work aims to determine whether the r-proteins are synthesised during extended stationary growth phase by calculating the changes in quantities of the r-proteins. This is a part of the bigger research on the topic of the extra ribosomal functions of r-proteins during stationary growth phase.

1 LITERATURE REVIEW

1.1 The structure of 70S ribosome

The ribosome in *Escherichia coli* is a ribonucleoprotein complex composed of r-proteins and rRNAs (Beer *et al*., 1960). It is assembled into the small ribosomal (30S) subunit, containing 16S rRNA molecule and 21 r-proteins, and the large ribosomal (50S) subunit, containing 23S and 5S rRNA molecules with 33 r-proteins, as shown in Figure 1 (Melnikov *et al*., 2012; Berg *et al*., 2002). The subunits then form the 70S ribosome (Melnikov *et al*., 2012). The rRNA/r-protein ratio in bacterial ribosomes equals to approximately 2:1 (Melnikov *et al*., 2012). The main function of the ribosome is the synthesis of proteins; a process that consists of four steps: initiation, elongation, termination, and recycling that must be completed successfully to make a protein (Dunkle and Cate, 2013).

The initiation step includes a specific initiator methionyl transfer RNA (tRNA) and the messenger RNA (mRNA) binding to the small ribosomal subunit (Cooper, 2000). The large ribosomal subunit then associates with the components, forming a 70S initiation complex that is ready to proceed the elongation of the polypeptide chain during the elongation step of the translation (Cooper, 2000). An active ribosome contains three binding sites for tRNAs: the aminoacyl-tRNA site (A site), peptidyl-tRNA site (P site), and exit-tRNA site (E site) (Dunkle and Cate, 2013). The initiator methionyl tRNA is bound at the P site (Cooper, 2000). Afterwards, the elongation step begins. Firstly, the next aminoacyl tRNA binds to the A site by pairing with the mRNA codon (Cooper, 2000). Secondly, a peptide bond between amino acids of the initiator methionyl tRNA at the P site and the second aminoacyl tRNA at the A site can be formed (Cooper, 2000). Afterwards, methionine is transferred to the aminoacyl tRNA at the A site of the ribosome, forming a peptidyl tRNA at this position and leaving the uncharged initiator tRNA at the P site (Cooper, 2000). The next step translocates the peptidyl tRNA from the A site to the P site, and the uncharged tRNA from the P site to the E site (Cooper, 2000). The ribosome is then left with a peptidyl tRNA bound at the P site and an empty A site (Cooper, 2000). The binding of a new aminoacyl tRNA to the A site then induces the release of the uncharged tRNA from the E site, leaving the ribosome ready for insertion of the next amino acid in the growing polypeptide chain (Cooper, 2000). Elongation of the polypeptide chain continues until a stop codon is translocated into the A site of the ribosome (Cooper, 2000).

The r-proteins have a great impact on the ribosomal functioning and assembly and, thereby, on the translational level of the cell in general. However, the interactions of the proteins and the structure of the ribosome are still being explored and examined. Therefore, the r-proteins have been of considerable interest among the scientific community.

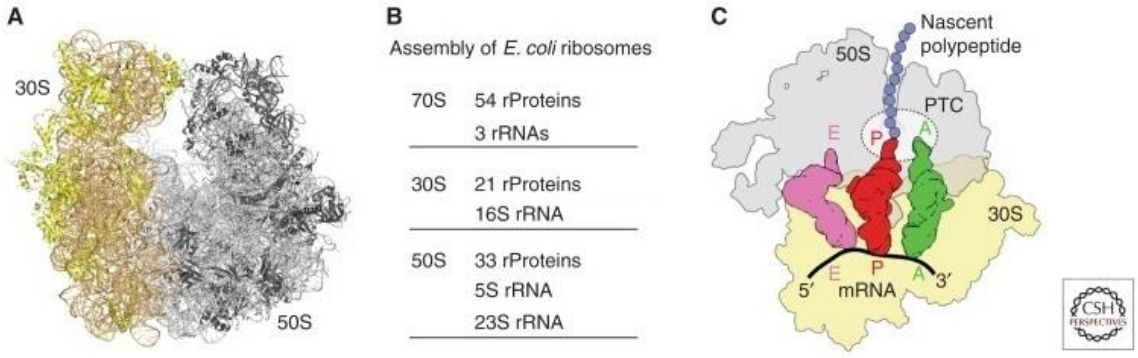

Figure 1. The prokaryotic ribosome (Arenz and Wilson, 2016). (*A*) Overview of the *Escherichia coli* 70S ribosome with 30S subunit coloured in yellow and 50S subunit in grey (Dunkle *et al*., 2011). (*B*) Table of assembly components of the 70S ribosome as well as the 30S and 50S subunits. (*C*) Schematic representation of the prokaryotic ribosome bound with three tRNAs showing the 30S subunit (yellow), 50S subunit (grey), A-tRNA (green), P-tRNA (red), E-tRNA (pink), nascent polypeptide chain (violet), and mRNA (black). The peptidyl transferase centre on the 50S subunit is depicted as a dashed sphere. (Arenz and Wilson, 2016)

1.2 Ribosomal proteins

The r-proteins play an important part in protein synthesis and the ribosome subunit assembly (Martín *et al*., 2003). Most r-proteins interact with rRNA, meaning that they contain RNA-binding domains. Therefore, the r-proteins are crucial for keeping the conformation of the ribosome and its structural integrity. It is believed that the role in locating and stabilising the rRNAs, thus facilitating the catalytic roles of ribosomes, makes r-proteins essential for the ribosome to function (Martín *et al*., 2003). In prokaryotes, the synthesis of many r-proteins is tightly regulated concerning nutritional conditions by translational feedback mechanisms, such as stringent response (Burgos *et al*., 2017; Nevskaya *et al*., 2006).

The stringent response is a mechanism caused by stress conditions, such as amino acid starvation (Burgos *et al.*, 2017). Such conditions result in the induction of the RelA synthetase of special alarmones, called ppGpp (Burgos *et al.*, 2017). The synthetase binds to an empty A-site of the ribosome, leading to the accumulation of ppGpp (Burgos *et al.*, 2017). The expression of the alarmone causes a decrease in the synthesis of r-proteins from the S10 and S20 operons by interacting with RNA polymerase that, in turn, regulates expression of genes (Burgos *et al.*, 2017). ppGpp also has a key role in regulating the expression of the genes that encode the proteins during the suppressed growth (Khmel', 2005). Thus, the entry into stationary growth phase is one of the conditions, due to which the stringent response is inducted.

Most of the r-proteins have a unique role and structure within the corresponding subunit and are necessary for the ribosome to successfully translate the mRNA into a correct protein. The nomenclature is used from (Ban *et al.*, 2014), where "b" stands for bacterial, "u" stands for universal, "S" stands for small ribosomal subunit, and "L" stands for the large ribosomal subunit protein.

There are 33 r-proteins in the large subunit and 21 r-proteins in the small ribosomal subunit. These proteins have various functions and the placements inside the ribosome. However, some of the r-proteins are still not fully explored. As of such, the important information about some r-proteins is described in the present work.

1.2.1 Large subunit r-proteins

uL2 is an r-protein of the 50S ribosomal subunit that binds to 23S rRNA and is localised near the intersubunit interface (Beauclerk and Cundliffe, 1988; Willumeit *et al.*, 2001). The protein changes its conformation, when the 50S subunit associates with the 30S subunit to form a 70S ribosome, by becoming more elongated (Willumeit *et al.*, 2001). uL2 is an essential element of a B7b bridge between the ribosomal subunits (Diedrich *et al.*, 2000; Willumeit *et al.*, 2001). It also is needed for such important functions as tRNA binding to both A and P sites and peptide bond formation (Diedrich *et al.*, 2000; Willumeit *et al.*, 2001). Moreover, it is believed that the protein belongs to a large multiprotein transcription complex that gathers at the RNA polymerase subunit, functioning as a direct activator throughout the specific binding to the subunit (Rippa *et al.*, 2010). It regulates the transcription by acting as an activator of rRNA operon transcription (Rippa *et al.*, 2010).

uL4 is a structural protein of the 50S ribosomal subunit and an autogenous regulator of both transcription and translation of its own operon, S10 (Singh *et al*., 2009). The S10 operon is the longest of the r-protein operons, coding for 11 r-proteins: uS10, uL3, uL4, uL23, uL2, uS19, uL22, uS3, uL16, uL29 and uS17 (Lindahl and Zengel, 1982). The overexpression of uL4 results in repression of the entire operon without significant effect on the synthesis of r-proteins from other operons, proving that the regulation is autogenous (Lindahl and Zengel, 1982). Moreover, the protein holds an extra ribosomal function of regulation of RNA degradation in *Escherichia coli* (Singh *et al*., 2009).

uL5 r-protein is an essential component of the large ribosomal subunit. It is involved in mediating the binding of 5S rRNA to 23S rRNA (Röhl and Nierhaus,1982). However, the binding is observed only in the presence of the uL18 protein (Röhl and Nierhaus,1982). Also, during the assembly of the 50S subunit of *Escherichia coli*, the protein uL5 plays a key role in the formation of the central protuberance of the subunit, the major component of which is 5S rRNA (Korepanov *et al*., 2012).

R-protein uL6 plays an important role in cell growth in *Escherichia coli* as it is an essential component of the large subunit that primarily binds to 23S rRNA during the late stage of the 50S subunit assembly (Shigeno *et al*., 2016). uL6 depletion results in a defect in the association of 50S subunits with 30S subunits (Shigeno *et al*., 2016).

uL11 is an r-protein in the 50S subunit that binds to 23S rRNA (Wimberly *et al*., 1999). The uL11-23S rRNA complex is involved in the regulation of the activities of elongation factors during the elongation cycle of translation (Yang and Ishiguro, 2001). Moreover, the protein acts as a stringent response regulator in *Escherichia coli* by controlling the RelA activity (Agrawal *et al*., 2018; Yang and Ishiguro, 2001).

The protein uL14 is a large subunit component of *Escherichia coli* ribosomes that binds directly to 23S rRNA (Morinaga *et al*., 1978). It is located on the intersubunit surface, forming the bridge B8 with the 30S subunit (Gao *et al*., 2003). uL14 bridge interaction region is close to the protein's rotation centre, resulting in a minor movement of the subunits in the correlation to the protein (Gao *et al*., 2003).

uL23 is a 23S rRNA binding protein, located at the exit of the peptide tunnel of the ribosome (Herold and Nierhaus, 1987; Kramer *et al*., 2002). The protein is essential for the growth of *Escherichia coli* as it interacts specifically with Trigger Factor and the ribosome (Kramer *et al*., 2002). In *E. coli*, Trigger factor (TF) is the chaperone that binds to unfolded polypeptides

at the beginning of biosynthesis (Ullers *et al*., 2003). It is the first chaperone to interact with the polypeptides, which is made possible by its specific interaction with uL23 (Ullers *et al*., 2003).

bL25 is a large ribosomal subunit r-protein that is capable of binding specifically to the 5S rRNA during the first step of 50S subunit reconstitution, regardless of other r-proteins (Chen-Schmeisserand and Garrett, 1977; Spierer and Zimmermann, 1978; Herold and Nierhaus, 1987). Its expression is stringently regulated at the transcription level (Aseev *et al*., 2015). Since bL25 is an RNA-binding protein, capable of recognising a specific fragment of 5S rRNA, it acts as an autogenous regulator of the *rpIY* gene at the translation level, defined as an r-protein repressor (Aseev *et al*., 2015).

bL28 is a rather basic component of the large subunit of *Escherichia coli* ribosomes that binds to the rRNA (Wittmann-Liebold and Marzinzig, 1977). bL28 protein is non-crucial for the assembly of the 70S ribosomes (Maguire and Wild, 1997). The absence of protein bL28 slows down the ribosome biosynthesis, which in turn slows down the growth rate of the cells, in comparison to the rapid growth when the protein is present (Maguire and Wild, 1997).

1.2.2 Small subunit r-proteins

bS1 is the largest of the r-proteins in *Escherichia coli* (Skouv *et al*., 1990). Low mRNA to ribosome ratio enhances the need for bS1 protein in the cell, which is required for an efficient translation of highly structured mRNAs (Moll *et al*., 2002; Skouv *et al*., 1990). Functioning as an mRNA binding molecule, the protein connects the 30S subunit with the messenger during protein synthesis (Moll *et al*., 2002; Skouv *et al*., 1990). bS1 can bind to the 30S ribosomal subunit only in the presence of the uS2 r-protein (Moll *et al*., 2002). The contact between bS1 and S2 are determined to be hydrophobic and electrostatic (Byrgazov *et al*., 2015). Moreover, the bS1 protein is essential for forming the translation initiation complex at internal ribosome binding sites (Moll *et al*., 2002; Skouv *et al*., 1990). The protein also has been suggested to assist in the positioning of the 30S subunit close to the translational start site (Moll *et al*., 2002). The depletion of bS1 for extended periods has been proved to initiate the starvation for amino acids, triggering the increase of synthesis of ppGpp. (Sørensen *et al*., 1998).

The protein uS4 acts as an initiator of 30S assembly, binding to the 5'-region of 16S rRNA (Nowotny and Nierhaus, 1988). It is one of the r-proteins that act as an autogenous regulator

of its own operon, α operon (Deckman *et al*., 1987). This operon codes for uS13, uS4, RNA polymerase α subunit and uL17 in *Escherichia coli* (Deckman *et al*., 1987).

uS9 is a small subunit protein that has a tail that interacts with the P-site tRNA (Hoang *et al*., 2004). The 30S P-site is responsible for binding and positioning the peptidyl-tRNA during polypeptide elongation and for maintaining the translational reading frame when the A-site is empty (Hoang *et al*., 2004). The mutant strains that lack uS9 have a slower growth rate, indicating that the tails may play a supporting functional role in translation (Hoang *et al*., 2004). The protein also has shown to contribute to the proper assembly of the 30S subunit (Hoang *et al*., 2004). The mutations in the uS9 protein cause the defects in 30S subunit function, which may affect the translational initiation, the only step, in which tRNA binds to the 30S P-site in the absence of 50S subunit (Hoang *et al*., 2004).

uS17 is a basic r-protein of *Escherichia coli* 30S ribosomal subunit (Yaguchi and Wittmann, 1978). It binds directly to the 5'-terminus of the 16S rRNA, thereby stabilising the RNA interactions (Held *et al*., 1974; Ramaswamy and Woodson, 2009).

1.3 Stationary growth phase

The growth of any bacterial culture represents a process of division of the cells in the culture to form two identical daughter cells (Pletnev *et al*., 2015). Plenty of nutrients provide the bacteria with a relatively fast growth rate (Kolter *et al*., 1993). However, the ability of the bacterial populations to initiate rapid growth by consuming nutrients implies that they are starved for most of the time (Kolter *et al*., 1993). This indicates that the cells remain in stationary growth phase for the better part of their lives. These cells can survive for extremely long periods in the absence of nutrients (Kolter *et al*., 1993). *Escherichia coli* cells cultivated for several days reveal a characteristic growth curve pattern made of five phases, as shown in Figure 2 (Pletnev *et al*., 2015).

The growth might be inhibited in response to starvation; however, it could be reinitiated by restarting the cycle once the nutrients are available again (Kolter *et al*., 1993). Therefore, when cells enter the nutrient-rich conditions after being in stationary growth phase, lag phase can be observed (Pletnev *et al*., 2015). It is characterised by an almost absence of bacterial growth in culture for some time, which might be connected to the need of adaptation of cellular metabolism to the new environmental conditions (Pletnev *et al*., 2015). Once the cells are adapted to the new conditions, they start to divide exponentially, entering an exponential growth phase (Pletnev *et al*., 2015). After the nutrients in the medium are

exhausted, as during exponential growth phase cells quickly use up the available nutrients and inhibit the exponential increase in biomass, bacterial culture enters stationary growth phase, which is characterised by a balance between the numbers of dividing and dying cells and represents a plateau in the growth curve (Kolter *et al.*, 1993; Pletnev *et al.*, 2015). Over time, cultures accumulate toxic products of catabolism in the environment, resulting in a decline in the number of viable cells, known as death phase (Pletnev *et al.*, 2015). After death phase, the better part of the population dies, and the dead cells release nutrients into the environment (Pletnev *et al.*, 2015). Residual cells may use these nutrients for their survival that would bring the bacterial culture to a state of long-term stationary growth phase (Pletnev *et al.*, 2015). After a long time of presence at stationary growth phase, the cells move into a nonculturable state with almost completely inhibited gene expression (Khmel', 2005).

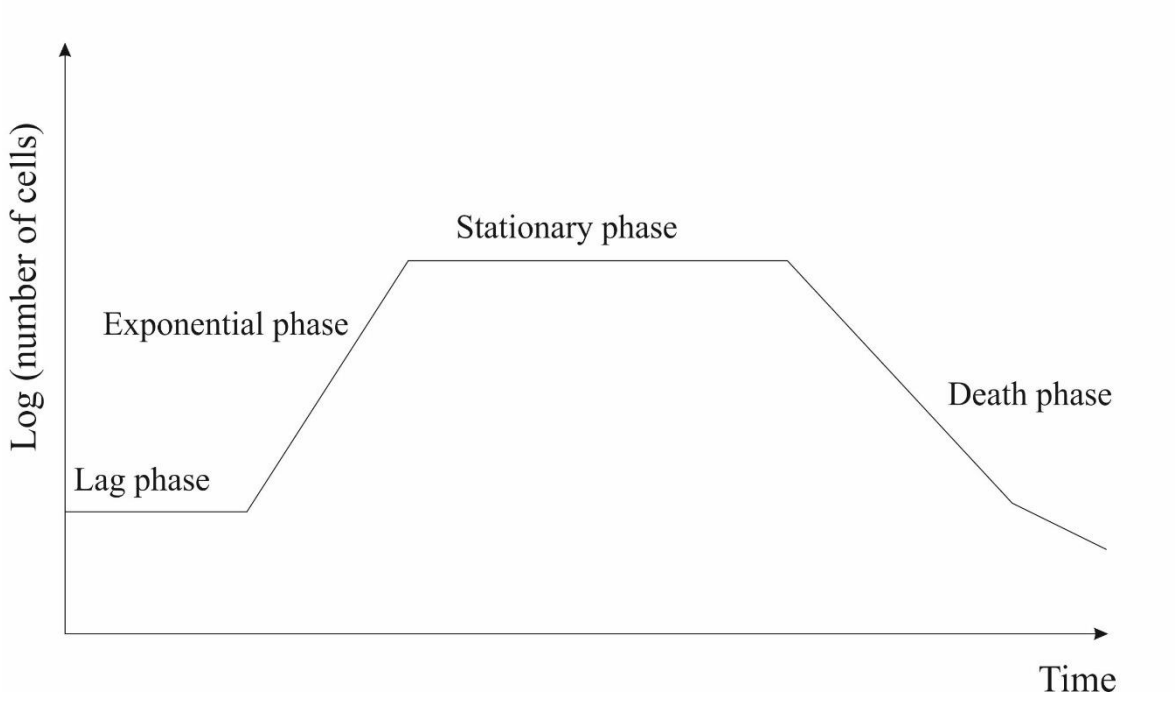

Figure 2. Growth phases of bacteria. The graph represents the change of the number of cells over time divided into five phases of growth: lag phase, exponential growth phase, stationary growth phase and death phase.

Escherichia coli cells that have been starved are more resistant to heat shock, oxidative stress, and osmotic challenge than exponential growth phase cells (Kolter *et al.*, 1993). The familiar rod shape of growing *Escherichia coli* cells is also changed in stationary growth phase, becoming smaller and almost spherical as the result of cell divisions without an

increase in cell mass due to starvation (Kolter *et al*., 1993). Such size reduction may improve a strain's survival by increasing cell numbers (Kolter *et al*., 1993).

1.3.1 Stationary growth phase effect on the gene expression

Stationary growth phase affects all the steps of the gene expression, starting from the transcription of mRNA and resulting in the translation of protein.

Transcription is a central and vital process when RNA is being synthesised on a DNA template (Pletnev *et al*., 2015). Under the conditions of stationary growth phase, a cell has to regulate transcription to activate the expression of the genes, required for survival under stress conditions, and to suppress the transcription of "unnecessary genes" (Pletnev *et al*., 2015).

Translation, or protein synthesis, is one of the most important processes in the cell, which is managed by the ribosome (Pletnev *et al*., 2015). In stationary growth phase, however, there is a rapid decrease in the level of protein synthesis, since translation is considered the most energy-consuming process in the cell (Pletnev *et al*., 2015). Due to the starvation and deficiency of amino acids in a bacterial cell, it is necessary for the cell to suppress translation (Pletnev *et al*., 2015).

1.4 Ribosome hibernation

The synthesis of ribosomes is a highly energy and resource-consuming process (Pletnev *et al*., 2015). Therefore, the suppression of translation for starved cells is required in order to retain an existing ribosome until the starvation is relieved (Pletnev *et al*., 2015). Such a process for storing an inactive ribosome, referred to as ribosome hibernation, is activated within the stringent response at the beginning of stationary growth phase (Pletnev *et al*., 2015). When *Escherichia coli* cells enter stationary growth phase, the composition of ribosomes is changed (Khmel', 2005; Lilleorg *et al*., 2018).

The ribosome hibernation makes changes in the translation machinery, using the active 70S ribosomes to form 70S inactive monomers and 100S dimers (Khmel', 2005; Pletnev *et al*., 2015). In *Escherichia coli*, there are two main proteins involved in the formation of 100S dimers, called ribosome modulation factor (RMF) and the hibernation promoter factor (HPF) (Khmel', 2005; Pletnev *et al*., 2015). The RMF protein's concentration starts to increase only upon the transition from exponential to stationary growth phase, having an inverse relationship between the level of expression of the *rmf* gene and the cell growth rate

(Khmel', 2005; Lilleorg *et al*., 2018). During the first step, the protein binds to each of the 30S subunits of the two 70S ribosomes (Pletnev *et al*., 2015). It is important that the protein is not able to bind to the translating ribosomes in order to prevent the formation of incomplete proteins that would result in being toxic to a cell (Pletnev *et al*., 2015). The second step involves the HPF protein binding to the 90S dimer to stabilise the structure and close the access for tRNA to the A- and P-sites of the ribosome (Pletnev *et al*., 2015). The resulting complex of two 70S ribosomes with HPF and RMF proteins is called 100S dimer, which is believed to be needed to preserve ribosomes (Pletnev *et al*., 2015). The dimerisation of two 70S ribosomes is believed to occur due to contacts between their 30S subunits (Khmel', 2005).

A ribosome-associated inhibitor A (RaiA) is a protein that promotes the formation of translationally inactive 70S monomers and prevents the recycling of ribosomes for translation initiation (Polikanov *et al*., 2012). Although RaiA and HPF proteins share similarities in their sequences, they have different effects on 100S dimer formation (Polikanov *et al*., 2012). HPF converts 90S into 100S particles, whereas RaiA prevents RMF-dependent 90S formation (Polikanov *et al*., 2012). Whenever the cells in stationary growth phase enter a nutrient-rich environment within a fresh medium, the complex of hibernation factors and ribosomes dissociates back into two active 70S ribosomes, which is essential for the successful active cell growth (Khmel', 2005; Pletnev *et al*., 2015).

2 THE AIMS OF THE THESIS

The main focus of the present work is to determine, whether the r-proteins of *Escherichia coli* are synthesised during extended stationary growth phase. Therefore, to identify the synthesised proteins, the following more specific aims were outlined:

- Growing *Escherichia coli* cells into extended stationary growth phase;
- Quantification of the change in r-protein amount in the cell proteome during extended stationary growth phase.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Materials

Escherichia coli strain MG1655-SILAC (MG-SILAC) parameters: F-, λ-, *rph*-1, *ΔlysA*, *ΔargA* (laboratory stock).

Harvested MG-SILAC cells, containing "medium-heavy" arginine (Arg6) and lysine (Lys4) (laboratory stock).

Unlabelled amino acids: 20 standard amino acids (Applichem).

Labelled amino acids: Arg10 – $[^{13}C]_6H_{14}[^{15}N]_4O_2$ (Silantes); Lys8 – $[^{13}C]_6H_{14}[^{15}N]_2O_2$ (Silantes); Arg6 – $[^{13}C]_6H_{14}N_4O_2$ (Silantes); Lys4 – $C_6H_{10}[^{2}H]_4N_2O_2$ (Silantes).

MOPS medium was based on the protocol from (Neidhardt *et al*., 1974) with changes in the amino acids composition and the concentration of glucose (20%). Heavy MOPS medium contains "heavy" arginine (Arg10) and "heavy" lysine (Lys8).

Luria Bertani (LB) medium was based on the protocol from (Sambrook and Russel, 2001).

The following equipment was used: safety cabinet (ScanLaf Mars Safety Class 2), incubation shaker (Infors HT Multitron), microcentrifuge (Heraeus Biofuge Fresco).

3.1.2 Bacterial strains and culture conditions

The cell cultures were labelled by Stable Isotope Labeling by Amino acids in Cell culture (SILAC) method. Two biological replicates of *Escherichia coli* MG-SILAC strain were used in the present work. Bacteria were cultured onto LB agar plates and incubated at 37°C overnight. Then, the cells were recultured into the fresh heavy MOPS medium, supplemented with "heavy" arginine and lysine. The cells were diluted into the fresh medium and incubated in the shaker at 37°C overnight three times to make sure that all the cells contained the "heavy" arginine and lysine. Afterwards, the culture was diluted into the fresh heavy MOPS medium and incubated in the shaker at 37°C for 24 hours. Then the cell culture was supplemented with 20 times excess of unlabelled arginine and lysine. The culture was incubated at 37°C in the shaker for 14 days. The harvested cells were collected at the 1st, 2nd, 6th, 10th and 14th days after the start of the growth by centrifugation (2376 g for 10 min) and stored at -80°C. The bacterial growth was observed spectrophotometrically at 600

nm at each of the time points. The samples were analysed using quantitative mass spectrometry.

3.1.3 Preparatoin of mass spectrometric samples

Collected samples were sent to the Proteomics Core Facility of the University of Tartu for quantitative mass spectrometry analysis of the proteome, where the cells were lysed and their A_{280} was measured. The samples were mixed in 1:1 ratio (A_{280}) with the standard. The MG-SILAC strain, containing "medium-heavy" arginine (Arg6) and lysine (Lys4), was used as the standard.

3.1.4 Mass Spectrometry analysis

The workflow is presented in Figure 3. The raw mass spectrometric data were analysed via MaxQuant (v1.5.6.0), Perseus (v1.6.2.3), Mascot search engine against UniProt *E.coli* database (29.10.2019) and Skyline freeware (v3.6). In the case of using Mascot search engine, the search was run against a hundred of the most intensive peaks in the dataset. The search parameters for all programmes were: peptide mass intolerance ±8 ppm and fragment intolerance ±0.6 Da. The enzyme was set to Trypsin/P. Carbamidomethylation of cysteine was set as a fixed modification. Arg6, Arg10, Lys4, Lys8 and oxidation of methionine were set as variable modifications. Afterwards, the data was organised in Excel and visualised using GraphPad (version 7.00). Finally, the results from two different software were compared and combined.

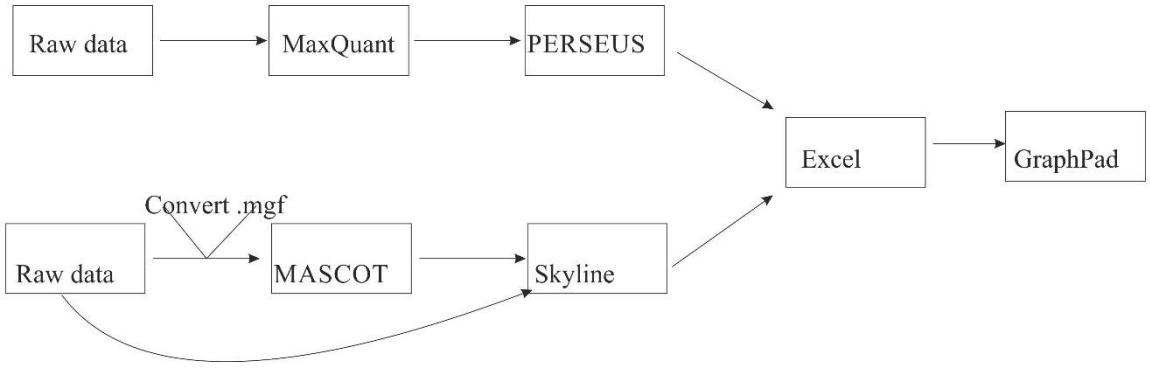

Figure 3. The pathway of analysis of the proteins. The graph represents an order of different applications used throughout the analysis of the protein quantities. The raw data obtained from the mass spectrometry was processed using MaxQuant, Mascot, Perseus and Skyline applications. The data was organised using Excel and displayed by GraphPad.

By the end of the analysis of the proteins, “light” (unlabelled) protein intensity to “medium-heavy” protein intensity SILAC ratio (L/M) was calculated for each protein from mass spectrometric data using the “medium-heavy” label as an internal standard. Finally, the difference in L/M ratio between time points shows the change in protein quantities over time.

3.2 RESULTS AND DISCUSSION

3.2.1 Growing of *Escherichia coli* cells in extended stationary phase

The cells were grown at 37°C for 14 days in extended stationary growth phase. The samples were collected from the cell culture and the OD_{600} was measured every other day. The spectrophotometric data in Figure 4 shows minimal changes in the optical density of cell culture, representing a steady curve. This allows to conclude that the balance between the number of dividing and dying cells was achieved, indicating stationary growth phase.

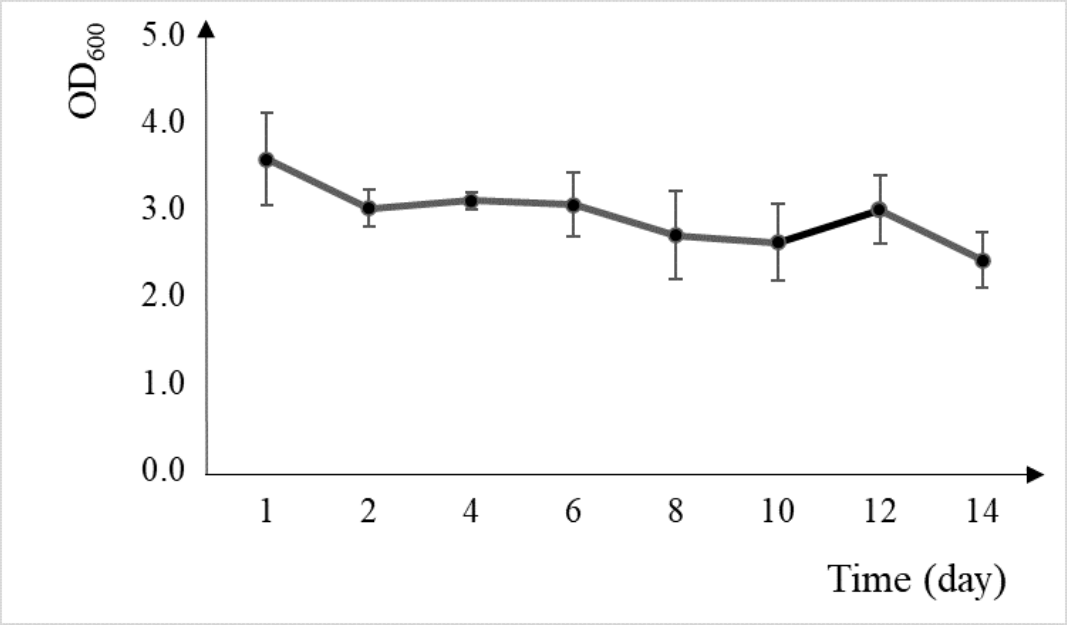

Figure 4. The optical density of the samples throughout stationary growth phase. The cells were grown in the MOPS medium into prolonged stationary growth phase and then collected every second day. The OD_{600} was measured at each time point, the mean of two replicates and the standard error were calculated and displayed as the relatively steady curve.

3.2.2 Analysis of the change in r-protein quantities

The cells were collected on the first, second, sixth, tenth and fourteenth days and analysed using quantitative mass spectrometry. The ratio between the light labelled protein intensity and the medium-heavy labelled protein intensity was calculated to determine the change of the amount of the proteins over time. 48 out of 54 r-proteins from the dataset were quantified, except bL31, bL34, bL35, bL36, uS8 and uS12. The difference in the r-protein quantities between the 1st and the 10th days was calculated and presented in Table 1.

From the quantified r-proteins, plenty of them were found to have a positive change in L/M ratio, indicating that the proteins were synthesised during stationary growth phase. We considered a 10% change of L/M ratio significant enough to be classified, synthesised or degraded (Table 1, footnote 2). Ribosome large subunit proteins uL2, uL4, uL5, uL6, uL10, uL11, uL14, uL18, bL21, uL22, uL23, bL25, bL28, uL29 and uL30 exhibited a change in L/M ratio during stationary growth phase (Table 1a). The small ribosomal subunit proteins, including bS1, uS2, uS3, uS4, uS5, uS9, uS10, uS17 and bS18, were also synthesised (Table 1b). L/M ratio was decreasing for only two proteins - uL16 and bS16, indicating the degradation during 10 days of cultivation. The results of the rest of the proteins were recognised inconclusive (Table 1, footnote 3). More data are needed for the determination of degradation and synthesis of these proteins.

Table 1. Colourmaps of the difference in quantities of proteins between the first and tenth days.

(a)

	Biological replicate 1	Biological replicate 2
uL1	1.41	1.09
uL2	1.85	1.13
uL3	1.77	1.08
uL4	1.90	1.30
uL5	1.49	1.21
uL6	1.32	1.22
bL12	0.93	1.11
bL9	1.20	1.07
uL10	1.13	1.61
uL11	1.80	1.47
uL13	1.31	1.08
uL14	1.32	1.28
uL15	1.35	0.94
uL16	0.75	0.51
uL17	1.62	1.09
uL18	1.53	1.29
bL19	1.77	1.05
bL20	1.39	0.74
bL21	1.30	1.27
uL22	1.22	1.12
uL23	1.74	1.34
uL24	1.41	0.89
bL25	1.43	1.18
bL27	1.15	0.84
bL28	1.61	2.19
uL29	1.75	1.39
uL30	1.56	1.41
bL32	1.29	1.03
bL33	0.97	0.66

(b)

	Biological replicate 1	Biological replicate 2
bS1	1.28	1.10
uS2	1.46	1.19
uS3	1.16	1.31
uS4	1.24	1.14
uS5	1.56	1.28
bS6	1.09	1.08
uS7	1.30	1.03
uS9	1.14	1.23
uS10	1.35	1.12
uS11	0.97	0.84
uS13	0.98	0.81
uS14	1.42	0.86
uS15	1.10	1.08
bS16	0.55	0.88
uS17	1.77	1.11
bS18	1.44	1.45
uS19	1.44	0.63
bS20	1.19	0.95
bS21	1.15	1.04

(1) The numbers were obtained by dividing the tenth day L/M ratio by the first day L/M ratio, indicating the difference in quantities between these two time points, within two replicates separately. L/M ratio represents the proportion between unlabelled protein intensity and medium-heavy labelled protein intensity.

(2) The green colour indicates the possible synthesis of the protein (L/M > 1,1), whereas the red colour means degradation (L/M < 0,9).

(3) Ambiguous proteins, where the biological replicates were different from each other, and/or the proteins that did not change with the value near 1; are not coloured.

(4) (a) presents the large ribosomal subunit proteins data, whereas (b) shows the small ribosomal subunit proteins quantities.

The previous research of *Escherichia coli* proteome has shown that during the transition from exponential growth phase into stationary growth phase the r-protein quantity level drops (Soufi *et al*., 2015). Such a decrease in the amount of proteins suggests that most of them are degraded (Soufi *et al*., 2015). However, the current study demonstrates that some of the r-proteins are synthesised. Although the proteins are mostly degraded during stationary growth phase, some of them are still synthesised in small amounts. Leading to a new research topic of the reasons, why the cell would keep synthesising the certain proteins, and whether these proteins are essential for the cell or not. For instance, some of the r-proteins might have extra ribosomal functions, which could result in an increase in the quantities during stationary growth phase.

3.2.2.1 Regulatory r-proteins during extended stationary phase

Under the stationary growth phase conditions, a cell has to regulate transcription and translation to activate or repress the expression of the genes, which is required for survival under stress conditions (Pletnev *et al*., 2015). Few r-proteins that have regulatory functions were found to be synthesised, including uL4, uL10, bL25 and uS4.

The uL4 protein autogenously regulates the whole operon S10 by inhibiting the synthesis of the proteins (Lindahl and Zengel, 1982). The S10 operon is one of the longest operons, as it codes for uS10, uL3, uL4, uL23, uL2, uS19, uL22, uS3, uL16, uL29 and uS17 (Lindahl and Zengel, 1982). It was previously shown that in the case of an overexpression of the uL4 protein, the repression of the whole operon would follow (Lindahl and Zengel, 1982). Thereby, according to the results, if the uL4 protein keeps being synthesised during stationary growth phase, it might result in the synthesis of the subsequent proteins. However, only 8 out of 11 r-proteins were proven to be synthesised during stationary growth phase, which include uL2, uL4, uL22, uL23, uL29, uS3, uS10 and uS17 (Supplementary Figure S1b, d, t, u, z; Figure S2c, i, o), leading to the question whether or not regulation of the operon is complete and unanimous.

R-protein uL10 is also a part of a feedback regulation, that acts as a repressor at the translational level of the L10 operon of *Escherichia coli* (Climie and Friesen, 1987).

The bL25, encoded by the *rpIY* gene, is a ribosomal gene that acts as an r-protein repressor that regulates its own synthesis (Aseev *et al*., 2015). *rpIY* gene expression is regulated stringently at the transcription level (Aseev *et al*., 2015). bL25 is able to specifically down-regulate its own gene expression (Aseev *et al*., 2015).

uS4 protein acts as an initiator of 30S assembly (Nowotny and Nierhaus, 1988). It regulates the expression of the α operon, that codes for uS13, uS4, RNA polymerase α subunit and uL17 in *Escherichia coli* by binding to the α mRNA (Deckman *et al*., 1987).

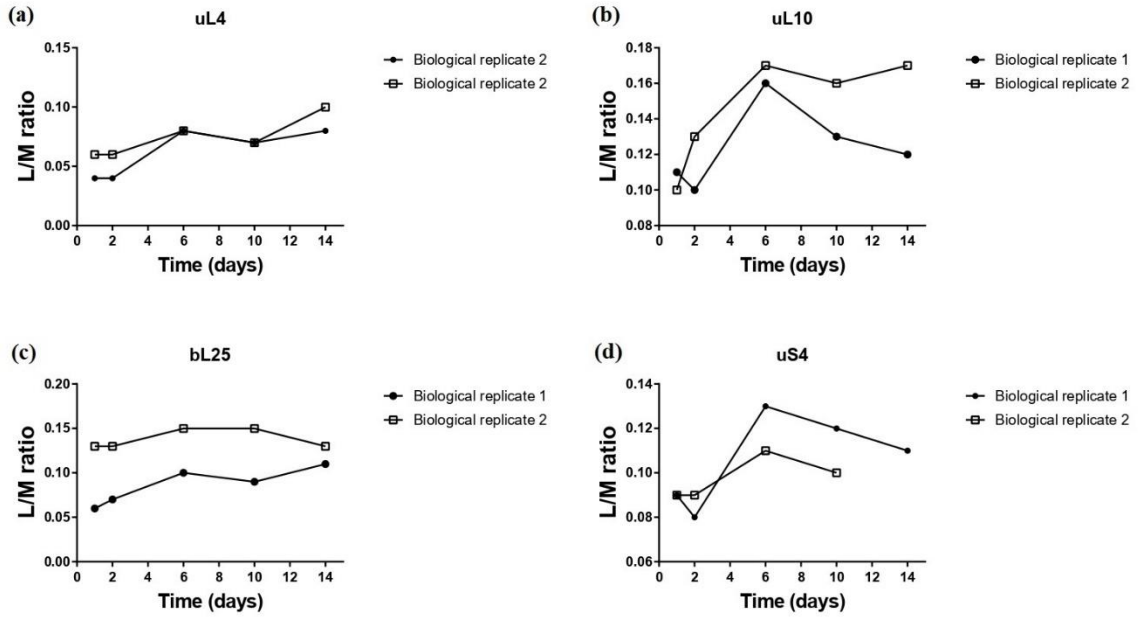

Figure 5. The synthesis of the uL4 (a), uL10 (b), bL25 (c) and uS4 (d) r-proteins. *Escherichia coli* cells were grown into stationary growth phase in the heavy MOPS medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis), indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines.

Regarding the Figure 5 results, the uL4, uL10, bL25 and uS4 r-proteins are proven to be essential components of the 70S ribosome during stationary growth phase, as they play a role in regulatory feedback mechanisms of their own operons by suppressing the synthesis of the rest of the r-proteins. It is necessary for the cells to inhibit the synthesis of proteins that are not vital in order to reduce energy consumption for survival. However, the question whether or not the whole operon is regulated unanimously remains open and requires further research of the subject.

3.2.2.2 Maintaining ribosome structural integrity during extended stationary growth phase

Most of the r-proteins keep the conformation of the ribosome by interacting with the rRNA molecules. The synthesis of many RNA-binding r-proteins is regulated by translational feedback mechanisms (Burgos *et al*., 2017; Nevskaya *et al*., 2006).

The results show that some of the r-proteins that were found synthesised during stationary growth phase have an ability to bind to rRNAs (Figure 6). These proteins include uL2, uL5, uL6, uL11, uL14, uL18, uL23, bL25, bL28, bS1, uS4 and uS17 (Supplementary Figure S1b, e, f, j, l, p, u, w, y; Figure S2a, d, o). The functions of the proteins vary, however, all of them have an ability to bind to the RNA molecules.

uL2 protein binds to 23S rRNA, playing an active role in the association of 30S and 50S subunits process, as it is an essential element of a bridge between the ribosomal subunits (Beauclerk and Cundliffe, 1988; Diedrich *et al*., 2000; Willumeit *et al*., 2001). uL5 and uL18 r-proteins are involved in mediating the binding of 5S rRNA to 23S rRNA during the assembly of the 50S subunit of *Escherichia coli*, and are dependent on each other (Röhl and Nierhaus, 1982). uL6 and uL14 also act at the large subunit. They bind to 23S rRNA during the 50S subunit assembly and, thereby, play an important role in cell growth (Morinaga *et al*., 1978; Shigeno *et al*., 2016). uL11 protein forms a complex with 23S rRNA, that is involved in the regulation of the elongation factors during the elongation cycle of translation (Yang and Ishiguro, 2001). bL25 protein binds specifically to the 5S rRNA during the first step of the large ribosomal subunit reconstitution (Chen-Schmeisserand and Garrett, 1977; Spierer and Zimmermann, 1978). bL28 plays a role in the 70S assembly by binding to the rRNA as well (Maguire and Wild, 1997; Wittmann-Liebold and Marzinzig, 1977). uS4 and uS17 both bind to 16S rRNA, after which uS17 stabilises the RNA interactions, whereas uS4 is needed for the 30S assembly initiation (Held *et al*., 1974; Nowotny and Nierhaus, 1988; Ramaswamy and Woodson, 2009; Yaguchi and Wittmann, 1978).

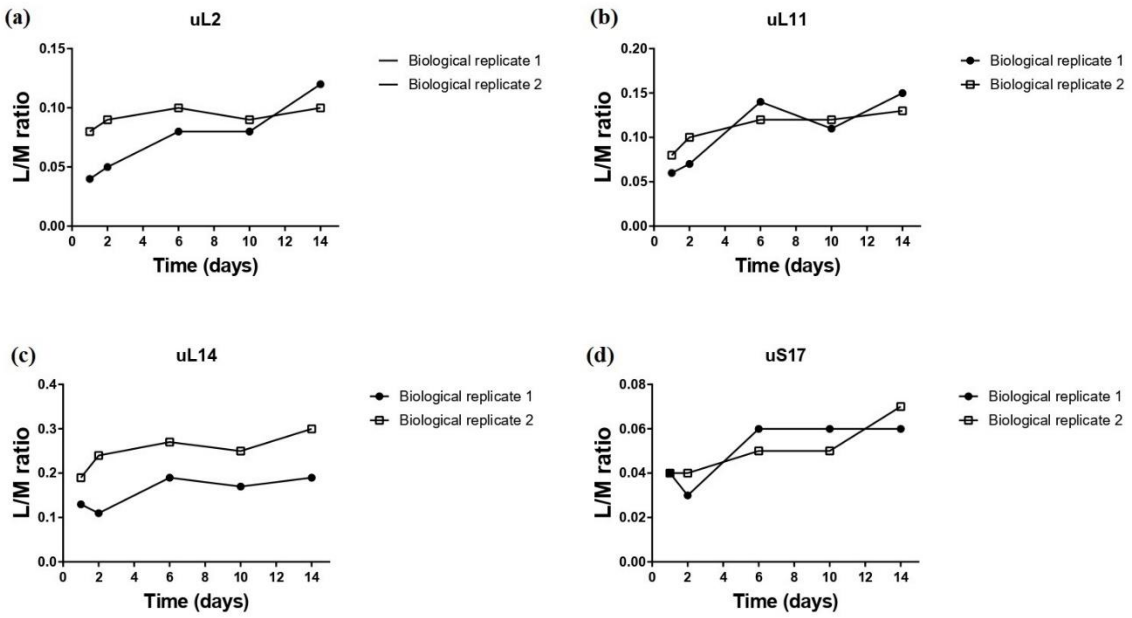

Figure 6. The synthesis of the uL2 (a), uL11 (b), uL14 (c) and uS17 (d) r-proteins. *Escherichia coli* cells were grown into stationary growth phase in the heavy MOPS medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis), indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines.

Therefore, the RNA-binding r-proteins were found to be synthesised during stationary growth phase. This synthesis might be required for the cell in order to survive the starvation periods of the cell growth due to the ability to maintain the structural integrity of the ribosome, for instance, via protein exchange. This feature might be vital for the cell as it retains an existing ribosome until the starvation is relieved and the growth is reinitiated.

3.2.2.3 Possible transition into death phase

Some of the quantified r-proteins were found to have an unusual activity within the 14th day of the experiment, including bL9, uL11, bL12, uL16, bL20, bL21, bL27, bL28, bS1, uS2, uS3, bS6, uS7, uS9, uS10, uS13, bS16, uS17 and bS21 (Supplementary Figure S1h, j, g, n, r, s, x, y; Figure S2a, b, c, f, g, h, i, k, n, o, s).

All of the r-proteins mentioned above have had a rapid increase in L/M ratio after the 10th day, regardless of the changes in the protein quantities throughout the whole time period.

Such unexpected results might indicate that the cell culture entered death phase after the 10th day.

Death phase takes place after the cell culture remains in stationary growth phase for a prolonged amount of time. The culture accumulates toxic products of catabolism in the environment, resulting in a decline in the number of living cells (Pletnev *et al*., 2015). After the cells enter death phase, the better part of the population dies, releasing the remaining nutrients into the environment (Pletnev *et al*., 2015). The rest of the cells use these nutrients for survival that would extend stationary growth phase (Pletnev *et al*., 2015).

According to Figure 7, the most controversial results were observed within the uL16 and bS16 r-proteins that initially were identified as degraded. However, both of the proteins show a considerable increase after the 10th day of the experiment.

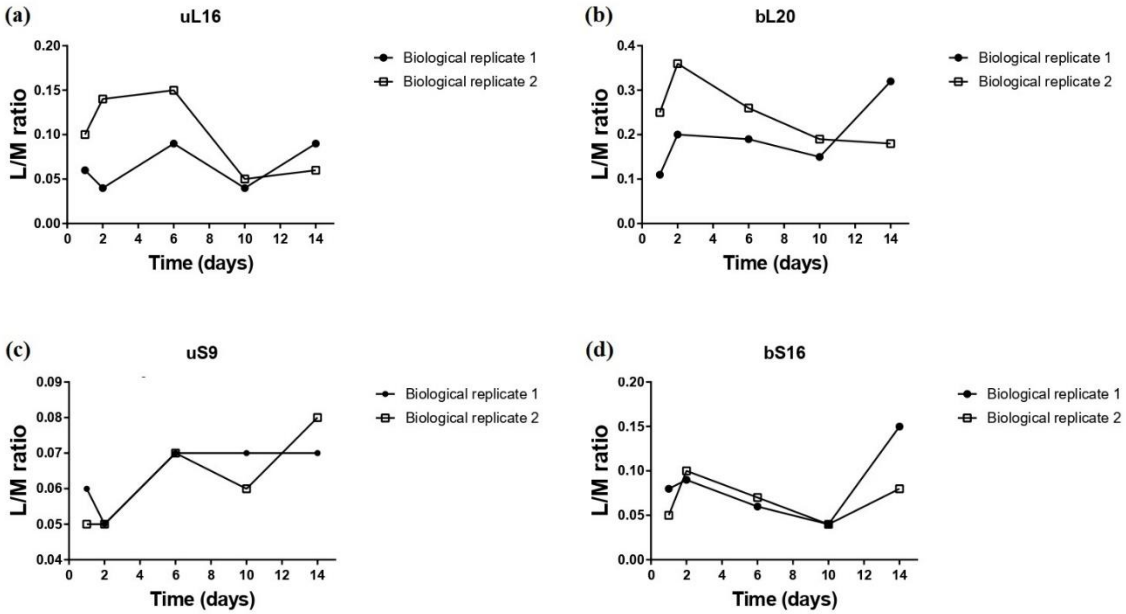

Figure 7. The change of the uL16 (a), uL20 (b), uS9 (c) and bS16 (d) r-protein quantities over time during extended stationary growth phase. *Escherichia coli* cells were grown into stationary growth phase in the heavy MOPS medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis), indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines.

This brings to a theory that the r-protein quantities begin to rise after the 10th day due to the cell culture entering death phase. The dead cells release the nutrients, which are later consumed by the surviving cells. The residual cells start synthesising the proteins at the expense of the new energy source, which would result in an increase of the level of the r-proteins after the 10th day of the experiment.

3.2.2.4 Ribosome-associated inhibition factor influence on the cell during extended stationary growth phase

The mass spectrometric data revealed quantities of the whole proteome of *Escherichia coli*. The protein RaiA, one out of three hibernation proteins, was found. It is known that the RaiA protein is antagonistic to HPF (Beckert *et al*., 2018). HPF enhances the 100S ribosome formation, whereas RaiA protein inhibits it (Ueta *et al*., 2005).

As can be seen from Figure 8, RaiA is synthesised prior to the 6th day (biological replicate 2) or 10th day (biological replicate 1), during which it drops drastically. Considering that at the beginning of stationary growth phase there are lots of the ribosomes in the cell, the RaiA synthesis, as one of the ribosome inhibiting factors, suppresses their translational activity. Since ribosomes are degraded during stationary growth phase (Piir *et al*., 2011), RaiA becomes unnecessary for the cell and starts degrading as well.

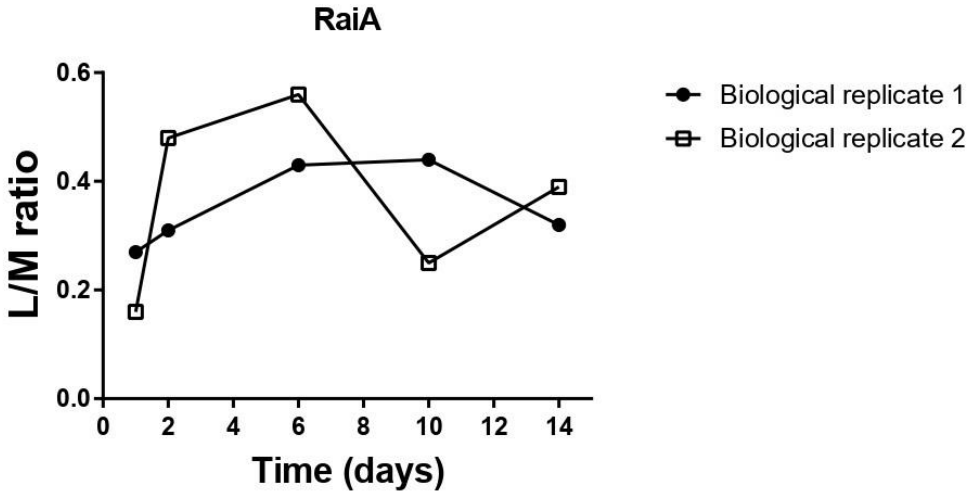

Figure 8. The change of the RaiA protein quantities over time during extended stationary growth phase. *Escherichia coli* cells were grown into stationary growth phase in the heavy MOPS medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis),

indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines.

Based on Figure 9, most of the r-proteins that were synthesised during the early stages of stationary growth phase were degraded rapidly after the 6th day, including uL3, uL4, bL9, uL10, bL12, uL15, uL16, bL21, uL24, bL27, bL28, uS2, uS4, bS6, uS9, uS14, bS18 and bS21 (Supplementary Figure S1c, d, h, i, g, m, n, s, v, x, y; Figure S2b, d, f, h, l, p, s). This might indicate that the amount of the ribosomes dropped extensively, resulting in the r-protein quantities reduction.

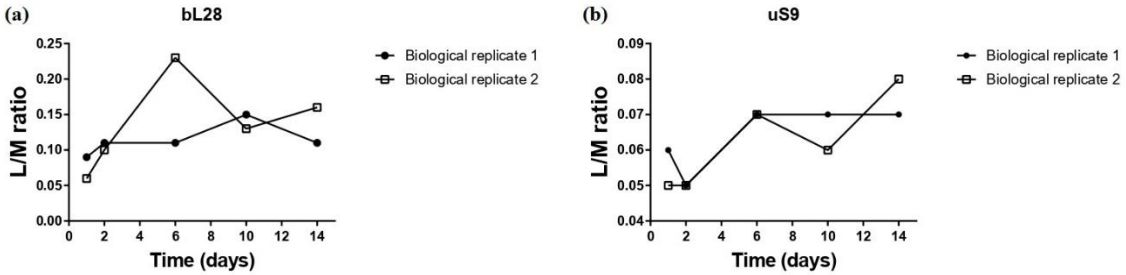

Figure 9. The change of the bL28 (a) and uS9 (b) r-protein quantities over time during extended stationary growth phase after the 6th day. *Escherichia coli* cells were grown into stationary growth phase in the heavy MOPS medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis), indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines.

Therefore, degradation of the RaiA after the 6th and 10th days may coincide with the degradation of the ribosomes. The cell needs to suppress the most energy-consuming processes during the early stages of stationary growth phase, for instance, translation through ribosome-associated inhibitor A. However, at the later stages of stationary growth phase the RaiA is degraded. Through which we theorise that the inhibition of the ribosomes through RaiA is not required in the later stages of stationary growth phase.

SUMMARY

Using the SILAC method, *Escherichia coli* cells were labelled during stationary growth phase. Afterwards, the quantities of the protein populations were determined. The results showed that some of the r-proteins are synthesised throughout extended stationary growth phase. 24 out of 48 quantified proteins kept being synthesised after the cells entered stationary growth phase. In order to survive, the cell has to conserve the energy by only producing the essential proteins. The found proteins might be synthesised due to their positive effect on the cell during stationary growth phase, such as the self-downregulation of the r-protein synthesis or retaining the structural integrity of the ribosome by RNA-binding proteins during starvation periods. However, the results of many of the identified r-proteins were found inconclusive, therefore, making it hard to determine whether or not they were synthesised. In order to get more details on the subject, further research is needed.

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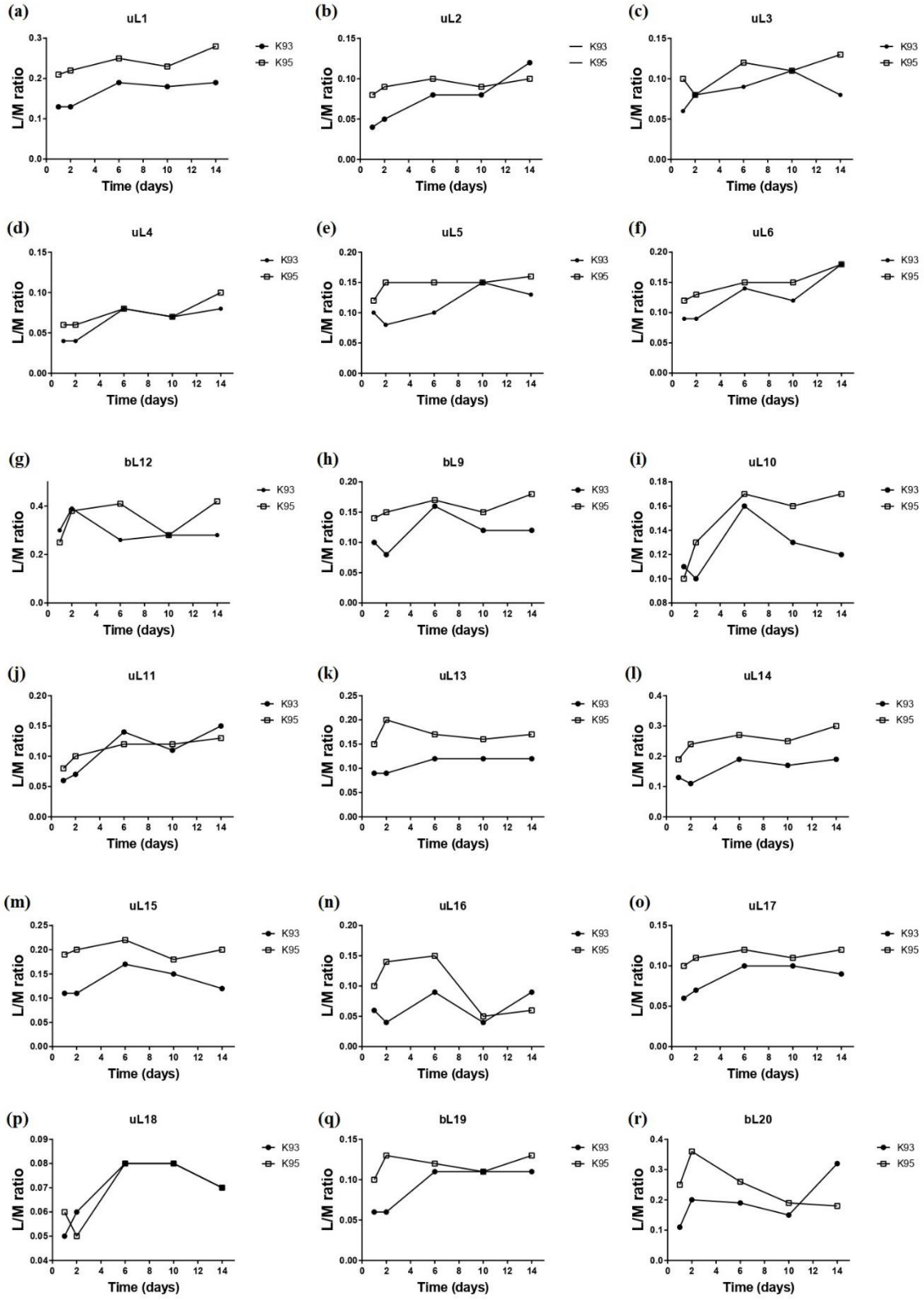
SUPPLEMENTARY MATERIALS



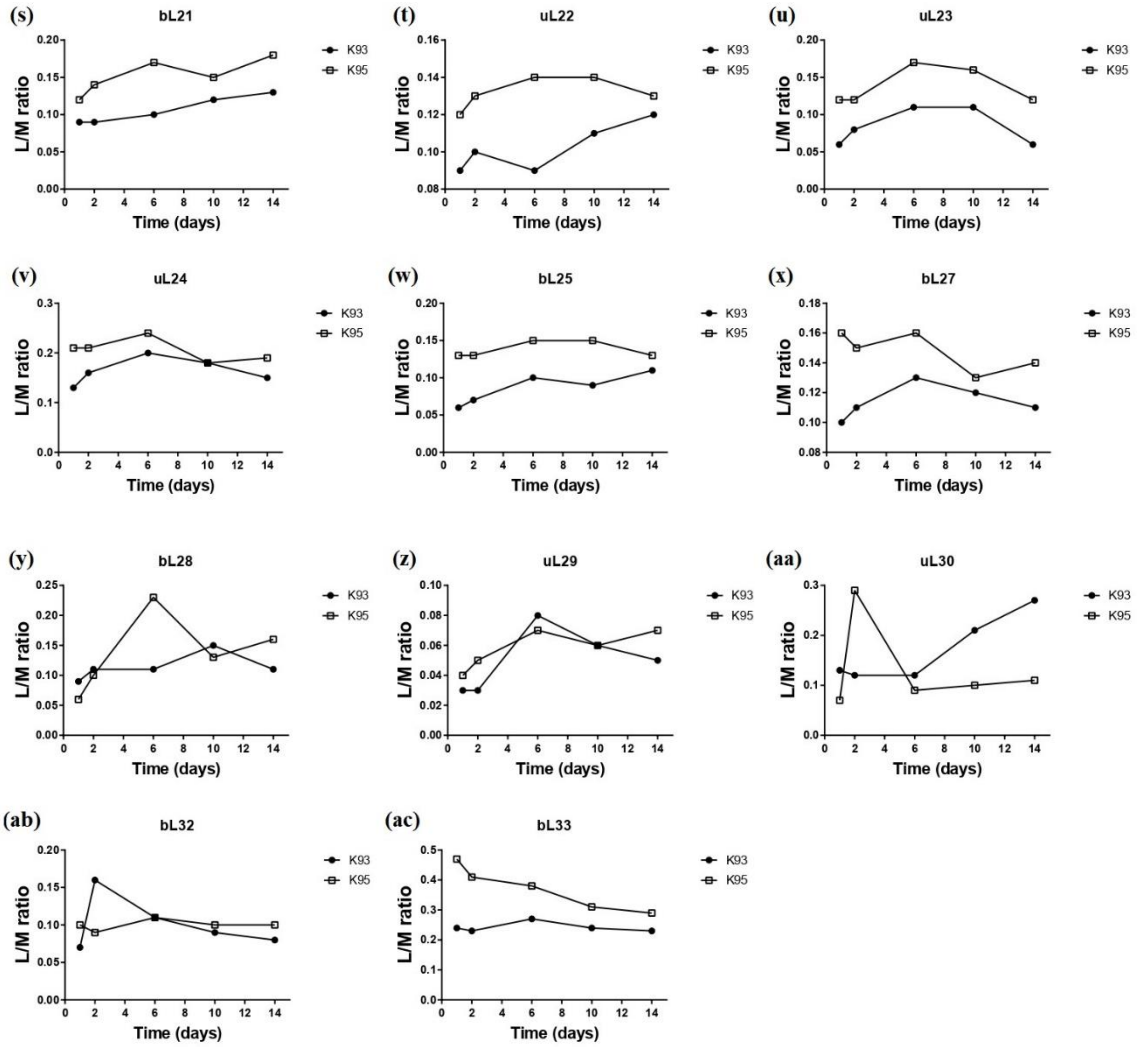
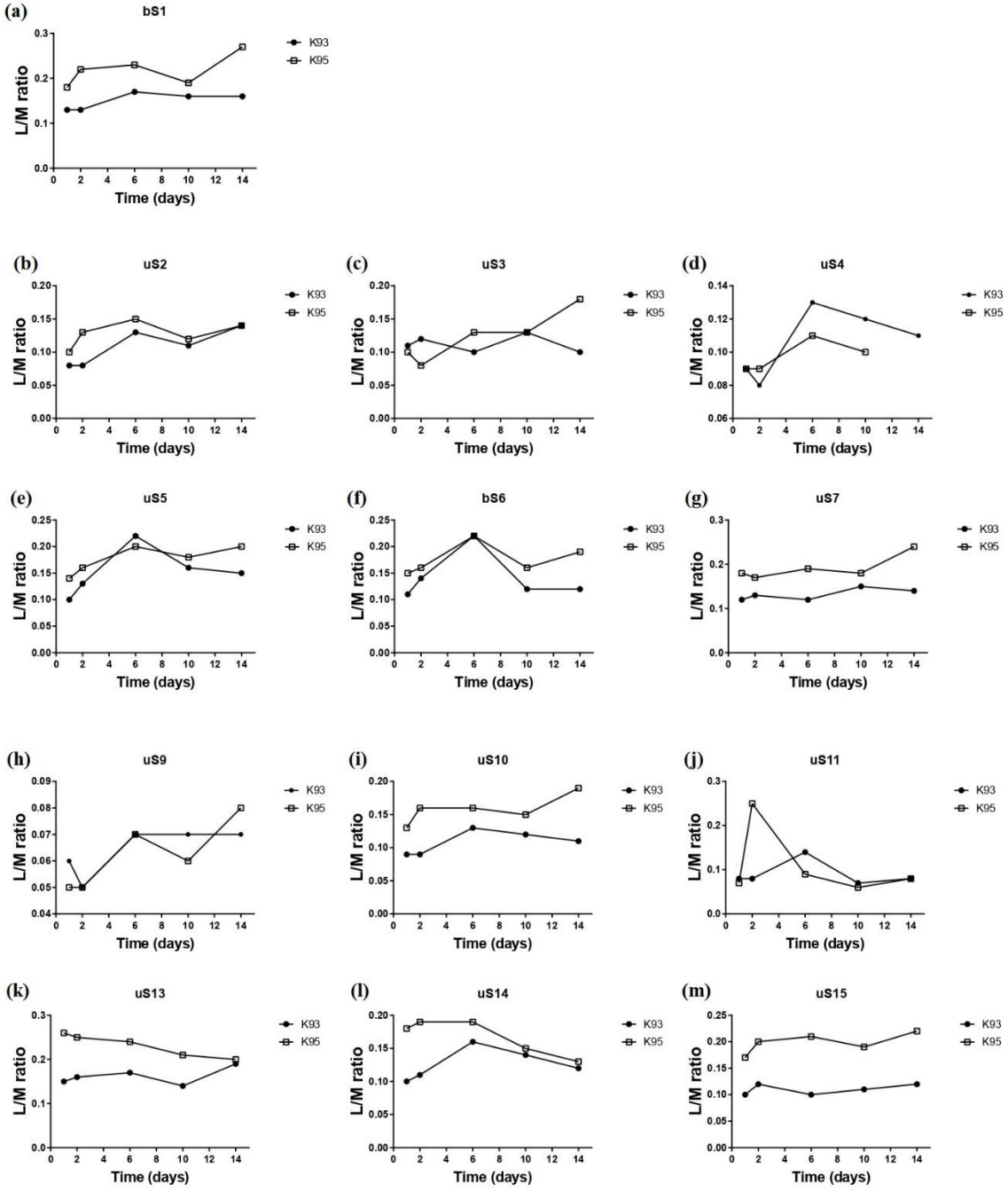


Figure S1. The change of the large subunit r-protein quantities during extended stationary growth phase. *Escherichia coli* cells were grown into stationary growth phase in the heavy medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis), indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines, where K93 represents biological replicate 1, and K95 represents biological replicate 2.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uS5
(f)
bS6
(g)
uS7
(h)
uS9
(i)
uS10
(j)
uS11
(k)
uS13
(l)
uS14
(m)
uS15
L/M ratio
Time (days)
K93
K95

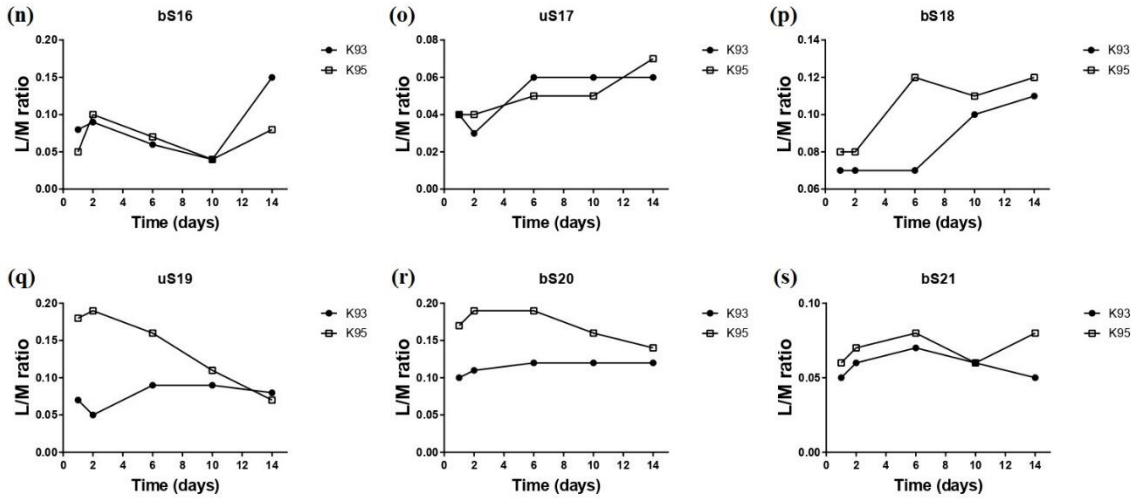

Figure S2. The change of the small subunit r-protein quantities during extended stationary growth phase. *Escherichia coli* cells were grown into stationary growth phase in the heavy medium. Afterwards, the cell culture was chased with an unlabelled or "light" amino acids. The samples were collected at each time point and mixed with the standard (medium-heavy amino acids) in 1:1 ratio. The graphs represent the change of L/M ratio (y-axis), indicating the quantity of the proteins, over time (x-axis). The data is represented by two biological replicates as separate lines, where K93 represents biological replicate 1, and K95 represents biological replicate 2.

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